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12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

We have evaluated the ability of tumor-seeking carrier-cells labeled with Thyl antibodies directly conjugated with AP. While many carriers were found in the tumor tissue at 12, 24 and 48 hours after injection, very little enzymatic activity was found in the malignant tissues. We conclude that although well retained on carrier-cell surfaces in vitro, even a directly conjugated antibody is "stripped" from the cells in vivo. We have found evidence that this happens during the cell's passage through the liver. We speculate that this might be due to macrophages, which are known to strip red blood cells for surface-attached immune complexes. Since this stripping is mediated via Fc-receptors on the macrophages, it is possible that F(ab')₂-fragments will not be stripped from the carrier-cell surface. We are now in the process of producing AP-labeled F(ab')₂-fragments of the Thyl.2 antibody. In addition, a biotin-based method for attaching AP to the carrier-cells is being tested. We believe that we, using carrier-cell labeled by one or both of these methods, will be able to deliver prodrug-activating enzymes selectively to breast cancer metastases.

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CONVERSION OF NON-TOXIC PRODRUGS TO ACTIVE, ANTINEOPLASTIC DRUGS SELECTIVELY IN BREAST CANCER METASTASES

SECOND ANNUAL REPORT

INTRODUCTION:

At time of diagnosis, breast cancer is often a systemic disease due to spread of malignant cells (metastasis) to other organs such as lungs, liver, bone marrow, and brain. While the primary lesion can often be successfully removed by surgical resection, treatment of the distant and often numerous metastases relies on systemic intervention. Systemic treatment of disseminated breast cancer is most often based on cytoreductive drugs, but damage to normal tissue limits the amount of cytotoxic drugs that can be safely administered to the patients. It is therefore very likely that the efficacy of most anti-cancer drugs can be improved if the amount of active drug in the malignant mammary tissue, especially the metastases, can be selectively increased compared to the normal tissues.

The purpose of this project is to develop and test a novel strategy for the treatment of breast cancer metastases, based on an innovative combination of chemotherapeutic and cellular immunological principles, which might substantially increase the anti-neoplastic efficiency of cytoreductive drugs, while at the same time reducing drug-induced damage to normal tissues. The idea is that if prodrug-activating enzymes are attached to tumor-seeking lymphoid cells before injection into tumor-bearing recipients, the cells will, due to their tumor-seeking capabilities (1-5), carry the activator selectively into the tumors. If a non-toxic prodrug is subsequently administered, the prodrug will be converted into active drug by the intratumoral prodrug-activator selectively in the tumor tissue. In this way, very high and therapeutically optimal levels of cyto-reductive drug can be generated in the metastatic breast cancer tissue. In contrast, very little active drug will be generated in the normal tissues, i.e., toxic side effects will be significantly reduced or even totally eliminated.

BODY OF PROGRESS REPORT:

The task for the third year of this project was, according to the original proposal (Task 4), to test the anti-tumor effect of AlkPhos/antibody/carrier-cell complexes and etoposide phosphate. However, as mentioned in the progress report for the second year,

we have had problems getting the enzyme to the tumors using a two-layer labeling of the carrier-cells (the enzyme was lost from the cell surface very fast in vivo). We therefore focused on conjugation of Thy-1 antibody (found to be the optimal antibody for labeling of the carrier cells) directly with the enzyme.

Task 2 A: Conjugation of the optimal antibody/antibodies (defined in Task 1) to alkaline phosphatase.

In year 2, we affinity-purified more than two liters of culture supernatant, giving us 16.2 mg antibody (at a concentration of 3.7 mg/ml). Some of this antibody has now been successfully conjugated to AP using the Maleimide Alkaline Phosphatase Conjugation Kit from Pierce.

By labeling A-NK cells with the conjugated antibodies and staining them with the substrate for AP and returning them to in vitro culture, we found that the antibodies were still present on the surface of the cells after 24 and 48 hours. Some of the label seemed to be internalized by the cells.

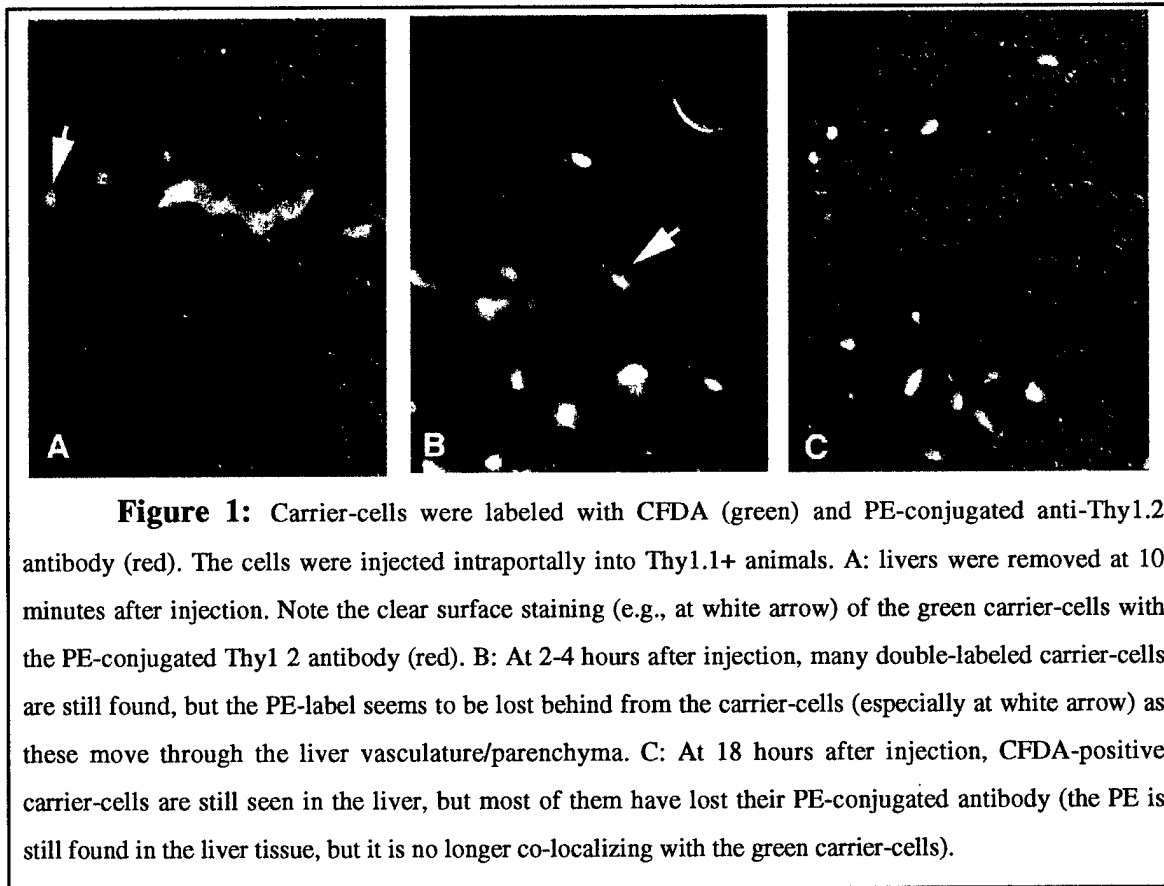
Task 2B: Evaluation of the tumor-seeking potential of the optimal AlkPhos/antibody/carrier-cells complex(es):

Having verified the stability of the Thy1.1 antibody-AP complexes on the surface of the carrier-cells, we injected cells 15 million cells labeled in this way into animals with lung metastases. Lungs were taken out at 12, 24 and 48 hours after injection. Frozen sections of the lungs were stained with PE-conjugated anti-Thy1.1 antibody. This staining revealed an impressive and highly selective accumulation of the carrier-cells in the lung tumors. More cells were found in the tumors over time, indicating either proliferation of the carrier-cells in the tumor or a continued localization to the tumors of circulating carrier-cells. The findings ensure that the AP-Thy1.1-label does not interfere with the tumor-homing potential of the carrier-cells.

Task 3. In vivo prodrug-converting activity of tumor-targeted AlkPhos/antibody/carrier-cell complexes:

Sections from the lungs were then stained with substrate for AP (BCIP/NBT). Quite disappointingly, very little positive staining was revealed by the AP substrate, indicating that the infiltrating carrier-cells had lost most of their label. Thus, using a directly conjugated antibody instead of a two-layer technique (as the previously attempted antibody-biotin-avidin-AP sandwich) did not solve this problem.

Last year, we showed numerous AP-positive cells in lungs and liver following BCIP/NBT-staining of lungs and livers removed from Thy1.1+ animals 1-2 hours after injection of CFDA and Thy1.2-avidin-AP double-labeled carrier-cells by the i.v. or intraportal routes. This showed that the AP enzyme is highly active in vivo (i.e., the lack of AP-staining was not due to inactivity of AP in vivo) Staining with goat-anti-rat antibody of tissue isolated from Thy1.1+ animals receiving Thy1.2-positive carrier-cells labeled with AP-Thy1.2 did not show any staining, strongly indicating that the anti-body was no longer present of the carrier-cells (i.e., the enzyme had not been cleaved from the antibody by plasma enzymes, leaving the antibody on the carrier-cells).

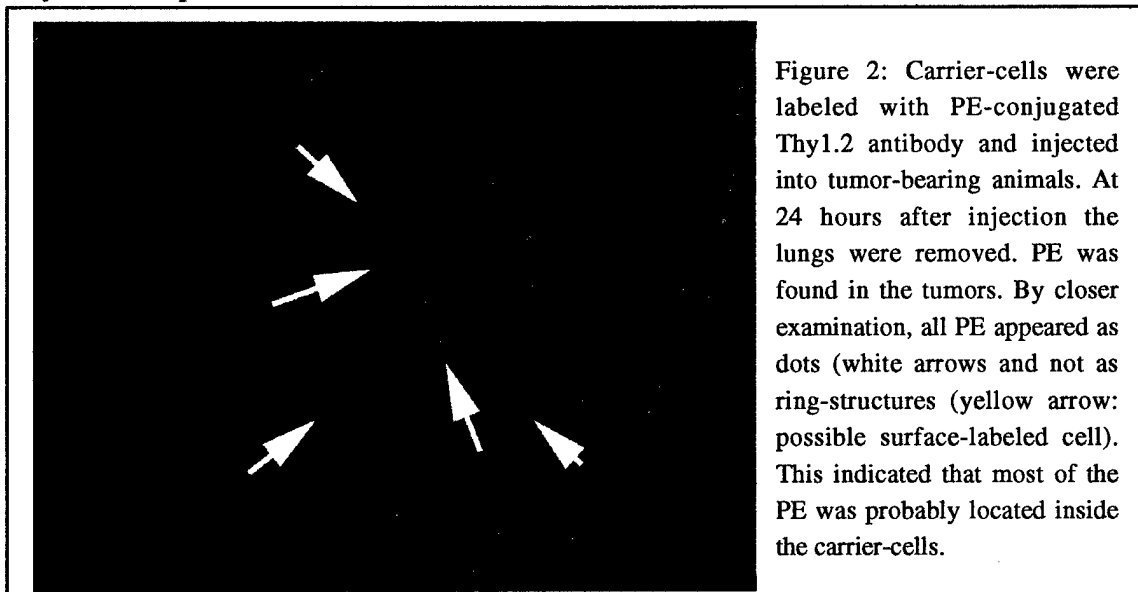


To further elucidate this phenomenon, we injected PE-anti-Thy1.2 labeled and CFDA-stained carrier-cells into Thy1.1+ animals by the portal vein. Livers were removed at 10 minutes, 2-4 and 18 hours after injection. At all times, CFDA-positive cells were found in the liver. However, only shortly after injection (10 minutes) was the PE-Thy1.1 label clearly associated with the carrier-cell membrane (Fig 1A). As early as 2-4 hours after injection, the PE-Label seem to be shed from the cells (Fig. 1B) and at 18 hours, most of the carrier-cells did no longer carry any of the PE-label, which was still seen in

the liver, possibly phagocytized by liver macrophages. Based on these observations, we must conclude that antibody attached to the carrier-cell surface is rapidly shed/removed from the cell surface.

The mechanisms by which the antibodies are removed from the carrier-cell surface are unknown to us, but we speculate that Fc-receptor positive macrophages in the liver are partly responsible. Among the many responsibilities of macrophages is to remove immune-complexes from the surface of red blood cells. It is therefore likely that the AP-anti-Thy1.1 complex, which is attached to the carrier-cells via their hypervariable portion and with the Fc portion sticking out from the cell, is removed from the carrier-cells by this mechanism. This problem should be solved by using F(ab')₂-fragments of the antibodies (with no Fc portion). We are now in the process of making AP-conjugated F(ab')₂-fragments of the Thy1.2 antibody.

If the antibodies are efficiently removed from the carrier-cell surface by macrophages, how come that we, as described in last year's report, have been able to find PE in tumors following i.v. injection of carrier-cells labeled with PE-Thy1.2 antibody? We now believe that this can happen, namely when a significant proportion of the PE-conjugated antibody becomes internalized before or shortly after injection of the carrier-cells. Once inside the cells, the antibody can no longer be removed by macrophages. This was confirmed by the fact that by re-examination of the pictures of tumors containing PE, we found that the PE in the tumors presented itself as dots (Fig. 2), i.e., it did not seem to be associated with a cell surface (in which case the PE should have appeared as a ring-structure (as in Fig. 1A at white arrow)). We are now trying to force the cells to internalize as much of the AP-antibody as possible before injection, to see, if the carrier-cells in this way can transport the AP to the tumors.



The following sections contain proprietary information:

Alternatives: While we believe that F(ab')₂-fragments of antibodies are probably much more stable on the carrier-cell surface in vivo than full antibody, we have considered a non-antibody dependent method for attaching enzyme to the carrier-cell surfaces. We have found that we are able to biotinylate carrier-cell surface proteins very efficiently. After biotinylation, we can attach any avidin-conjugated compound directly to the biotin. Apparently, we can control the rate of internalization by the carrier-cells of the biotinylated proteins by the level of cross-binding we generate after adding the avidin-conjugates to the cells (small amounts of avidin favors cross-binding and internalization). We are very excited by this discovery and we think that this method may turn out to be a very useful, safe and easy way of “loading” cells of all kinds with a variety of compounds. Experiments to evaluate the ability of biotinylated carrier-cells conjugated to AP-avidin to bring AP to tumors are in progress.

Key Research Accomplishments:

- Adoptively transferred, tumor-seeking A-NK cells can act as carrier-cells capable of transporting pro-drug-converting enzymes selectively into tumor tissue.
- Full length antibody can be used for the attachment of enzyme to carrier-cells. While the enzyme-antibody complex remains on the carrier-cell surface in vitro for ~10 hours (where after it slowly becomes internalized), it is stripped from the carrier-cell surface in vivo after a few hours.
- Stripping of antibody from carrier-cell surfaces occurs in the liver and may be prevented by using F(ab')₂-fragments instead of full length antibody containing the Fc portion.
- Biotinylation of carrier-cell surfaces may serve as an antibody-independent, cheap and easy method for the attachment of enzymes to carrier-cells.

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REPORTABLE OUTCOMES:

- Basse, PH: Targeted delivery of anti-neoplastic agents to breast cancer metastases (DoD BCRP meeting, Atlanta, June, 2000).
- A Patent covering targeted delivery of prodrug-activating enzymes by lymphoid carrier-cells will be applied for within the next 12 months.
- Based on work supported by this award, an application named: “A new strategy for targeted chemo-immunotherapy of cancer” has been submitted to the NIH (June 2000).

CONCLUSIONS:

Third year: We have demonstrated that carrier-cells labeled with antibodies directly conjugated to AP are not able to deliver significant amounts of AP to the tumors. We have provided some evidence that this is due to removal of surface-bound antibody by macrophages. We have also found indications that if the label is internalized before or shortly after injection of the carriers-cells, chances that the label are transported to the tumor by the carrier-cells increases.

As an alternative to antibody-dependent labeling of carrier-cells, we propose to directly biotinylate cell surface proteins and subsequently attach avidin-conjugated enzymes to the cells.

Fourth year - Planned experiments:

We will produce F(ab')₂-fragments of the Thy-1.2 antibody. These will be labeled with AP using the Maleimide Alkaline Phosphatase Conjugation Kit from Pierce. Carrier-cells will be labeled with the AP-conjugated F(ab')₂-fragments and injected into tumor-bearing animals. The ability of these carrier-cells to bring the AP to the tumor will be analyzed.

Carrier-cells will be labeled with biotin and subsequently with AP-conjugated avidin. The ability of these carrier-cells to bring the AP to the tumor will be analyzed. In particular, we will examine the ability of the carrier-cells to bring the AP to the tumors if the biotin-avidin-AP has been allowed to internalize before injection.

So what: As soon as we are able to demonstrate that prodrug-activating enzymes can be transported by carrier-cells selectively into mammary metastases, either using the F(ab')₂-conjugated carrier-cells or the biotin-based method, we will submit a patent application on this subject to the Office of Technology Transfer at the University of Pittsburgh. Immediately hereafter, we'll test the ability of the intratumoral AP to convert the prodrug etoposide-phosphate into active drug (etoposide) and the effect hereof on tumor growth (Task 4). We envision that one or several medical companies will become interested in this concept and help us to develop other enzyme-prodrug systems (in addition to the alkaline phosphatase/etoposide phosphate system) with high specificity for breast cancer cells.

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APPENDICIES:

N.A.